REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

I. Status Of The Claims

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Claims 1-54 and 58-78 are pending. Claims 1-8, 10-22, 43-46, 49-55, 58-71 and 73-78 were rejected. Claims 9, 23-30, 47-48 and 72 were objected to. The Examiner is thanked for indicating allowable subject matter.

Claim 17 is amended. Exemplary support for this amendment can be found, e.g., at original claims 1 and 17, and page 6, lines 1-3, of the application.

Upon entry of this amendment, claims 1-54 and 58-78 will be pending in the application. As the foregoing amendment does not introduce new matter, entry thereof by the Examiner is respectfully requested.

II. Claim Rejections -- 35 U.S.C. § 112, Second Paragraph

A. Claims 17, 58 And 60-63 For Reciting "One Or More Substitutions, Additions, Deletions, Or Modifications"

Claims 17, 58 and 60-63 were rejected under 35 U.S.C. § 112, second paragraph, for reciting the phrase "one or more substitutions, additions, deletions, or modifications." The Examiner stated that it was not clear where the substitutions, additions, deletions, or modifications occur in the sequence and how many amino acids are substituted, added, deleted or modified, or what the resulting sequence is. Applicant respectfully traverses this ground of rejection.

The breadth of a claim is not to be equated with indefiniteness. *In re Miller*, 441 F.2d 689, 169 USPQ 597 (CCPA 1971); *see also* MPEP § 2173.4. If the scope of the subject matter embraced by the claims is clear, and if applicants have not otherwise indicated that they intend the invention to be of a scope different from that defined in the claims, then the claims comply with 35 U.S.C. § 112, second paragraph. *See* MPEP § 2173.4.

The pending claims recite an analog of natural GLP-2 "having one or more substitutions, additions, deletions, or modifications." Applicant specifically teaches suitable substitutions, additions, deletions, or modifications for GLP-2 analogs, for example, at page 6, lines 1-12, of the Application. Moreover, Applicant specifically teaches and claims the functional characteristics of GLP-2 analogs. *See* page 5, lines 24-33, and page 6, lines 24-28, of the application. As such, the pending claims should be given their broadest reasonable interpretation consistent with the specification. MPEP § 2111. Read in light of the specification, a skilled artisan would understand the subject matter of the claimed invention. Accordingly, Applicant's claims are definite and this ground of rejection should be withdrawn.

B. Claim 59 For Reciting Amino Acid Substitutions Without Indicating A "SEQ ID NO:"

Claim 59 was rejected under 35 U.S.C. § 112, second paragraph, for reciting amino acid substitutions at various positions without indicating the "SEQ ID NO:" of the reference sequence. Applicant respectfully requests reconsideration and withdrawal of the rejection.

For compliance with the definiteness requirement of 35 U.S.C. § 112, second paragraph, the claim language must meet the threshold requirements of clarity and precision regardless "whether more suitable language or modes of expression are available." MPEP § 2173.02. The essential inquiry is whether the claims set out their subject matter with a reasonable degree of clarity and particularity. *See* MPEP § 2173.02. This inquiry is analyzed in light of: (1) the content of the particular application, (2) the teachings of the prior art, and (3) the claim interpretation that would be given by one of ordinary skill in the art at the time the invention was made. *See* MPEP § 2173.02.

Those skilled in the art would understand the metes and bounds of the claimed specific peptide analogs. The GLP-2 peptide was well-known to those skilled in the art at the time the invention was made. For example, a review article summarized much of the contemporaneous knowledge concerning the peptide:

> the GLP-2 sequence (Fig. 2) was detected in all isolated mammalian proglucagon cDNAs and genes (6, 7, 8, 9, 10) as a 33 amino acid peptide located carboxyterminal to GLP-1 and intervening peptide 2 (Figs. 1 and 2). Subsequent studies demonstrated that fish, chickens, and lizards generate GLP-2 in the gut as a result of tissue-specific alternative RNA splicing of proglucagon RNA transcripts (11, 12). In mammals, tissue-specific posttranslational processing liberates GLP-2 from proglucagon in the intestine and brain but not in pancreas, as a result of cell-specific expression of prohormone convertases in gut endocrine cells (13). Isolation and sequencing of GLP-2 from the porcine and human intestine confirmed that GLP-2 is a 33 amino acid peptide (Fig 2), corresponding to proglucagon 126–158, ending in a carboxyterminal Asp residue (14, 15).

D.J. Drucker, *J. Clin. Endocrinol. Metab. 86(4)*:1759-64 (2001). As exemplified by the above excerpt, those skilled in the art would recognize that *naturally occurring* GLP-2 is a 33 amino acid peptide cleaved from proglucagon and ending in a specific residue. This recognition is consistent with the disclosure of the application. *See, e.g.*, page 1, lines 20-25.

In addition and as previously stated, those skilled in the art would have recognized a number of specific GLP-2 analogs. *See* pages 17-19 of the Paper of September 7, 2004; *see also* U.S. Patent Nos. 5,789,379 and 5,952,301. Likewise, Applicant specifically teaches suitable GLP-2 analogs, for example, at page 5, line 24, to page 6, line 34, of the application.

In view of the disclosure of the application and the state of the art at the time the invention was made, those skilled in the art would recognize the subject matter recited as an "analog of natural GLP-2." Accordingly, Applicant respectfully requests reconsideration and withdrawal of the rejection.

III. Claim Rejections -- 35 U.S.C. § 103

A. Rejection Of Claims 1-8, 10, 22, 49-55, 58, 63-71, 73 And 78 As Being Obvious Over Knudsen In View Of Kornfelt

Claims 1-8, 10, 22, 49-55, 58, 63-71, 73 and 78 were rejected under 35 U.S.C. § 103(a) as being allegedly obvious over Knudsen et al. (WO 99/43361, "Knudsen") in view of Kornfelt et al. (U.S. Patent No. 5,652,216, "Kornfelt"). Applicant respectfully traverses and requests reconsideration and withdrawal of the rejection.

1. The Examiner's Basis for the Rejection

The Examiner asserts that it would have been obvious to a person of ordinary skill in the art to prepare a pharmaceutical composition of GLP-2 as indicated by Knudsen with the addition of histidine as a stabilizing agent as taught by Kornfelt because histidine had been shown to stabilize glucagon and GLP-2 is a glucagon-like peptide.

2. There is no Motivation to Combine the Teachings of Knudsen and Kornfelt Because Kornfelt Relates To Glucagon, Which is Significantly Different Than Applicant's Claimed GLP-2

A proper rejection under 35 U.S.C. § 103(a) requires two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition, or device, or carrying out the claimed invention, and (2) whether the prior art would also have revealed that in making or carrying out the claimed invention, those of ordinary skill would have a reasonable expectation of success. *Both the suggestion and the reasonable expectation of success must be found in the prior art, and not in the applicant's disclosure. See In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). In the present case, the Examiner has failed to establish a *prima facie* case of obviousness for the following reasons.

There is no teaching or suggestion in the cited art to combine the teachings of Knudsen with the teachings of Kornfelt to obtain the claimed invention. Kornfelt is directed to a pharmaceutical composition comprising glucagon and a stabilizing amount of a pharmaceutically acceptable ampholyte including, for instance, histidine. Despite similarities

in their names, glucagon and a naturally occurring GLP-2, or an analog thereof, are not interchangeable and have different properties, characteristics, and functionality.

For example, the physical properties of each protein are so different that it is not intuitive that excipients and formulations that stabilize glucagon would have the same effect on naturally occurring GLP-2, or an analog thereof. The following figure shows that there is *very little* structural equivalence between glucagon and GLP-2.

```
hGLP-2      HADGSFSDEMNTILDNLAARDFINWLIQTKITD
              +  + +       ++    + ++  ++  +
Glucagon      HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
```

Although the origin of the sequences are related, GLP-2 possesses only 33% sequence homology to glucagon – which is equivalent to 11 of 33 amino acid residues. Moreover, the chart provided below shows some of the properties, characteristics, and functionality which are different between glucagon and ALX-0600, an exemplary GLP-2 analog.

Property	**ALX-0600**	**Glucagon**
Soluble In H_2O?	Yes	No
Soluble At pH?	Above About 5.5	About 2.8
High Affinity Binding to GLP-2 Receptor?	Yes	No
In Vivo Intestinotrophic Activity?	Yes	No

As shown above, the solubility glucagon and GLP-2 are dramatically different. Applicant teaches that [Gly^2]hGLP-2 precipitates from solution below about pH 5.5. *See* page 7, lines 3-6, of the Application. In contrast, Kornfelt teaches that the preferred pH for glucagon is pH 2.8. *See* col. 3, lines 9-12, of Kornfelt. Likewise, glucagon is practically insoluble in water. *See* European Pharmacopoeia Commission, The European Pharmacopeia (2nd Ed.), page 612 (1989).

Moreover, glucagon and GLP-2 are functionally distinct. GLP-2 analogs that activate GLP-2 receptor signal transduction *in vitro* display intestinotrophic activity *in vivo*. *See, e.g.*, Munroe et al., *Proc. Natl. Acad. Sci. U.S.A. 96(4)*:1569-73 (1999) ("Munroe"). Glucagon does not. *See* page 1573, Table 2, of Munroe. Likewise, GLP-2, but not glucagon, is capable of high affinity binding to the GLP-2 Receptor. *See* page 1573, Table 2, of Munroe.

Accordingly, glucagon and a naturally occurring GLP-2, or an analog thereof, are not interchangeable as each peptide has different properties, characteristics, and functionality.

3. There is no Motivation to Combine the Teachings of Knudsen and Kornfelt Because of the Empirical Nature of Peptide/Protein Formulations

Additionally, a person of ordinary skill in the art would know that there are several problems that may be encountered in designing peptide/protein formulations due to their empirical nature.

In Applicant's previous response, several references were cited that detail the difficulties that may be involved with designing peptide/protein formulations. *See e.g.,* Pikal et al., *Pharm. Res. 8(4)*:427-436 (1991) ("Pikal") and Cleland et al., "Formulation and delivery of proteins and peptides: design and development strategies" in Cleland et al. (Eds.), Formulation and Delivery of Proteins and Peptides, American Chemical Society, Washington D.C., p. 1-19 (1994) ("Cleland"), Cleland prominently noted that "one parameter that impacts all the major degradation pathways is the solution pH." (*See e.g.*, page 5, first full paragraph). Additionally, Cleland states that:

> *[t]he design and production of protein and peptide drug formulations is not well developed and many of the mechanisms for stabilization and delivery of these drugs have not been determined . . . Each molecule has its own unique physical and chemical properties which determine its in vitro stability.*

Page 1. Because these inherent difficulties in designing peptide/protein formulations, one of skill in the art at the time the claimed invention was made would not have been motivated to attempt to make Applicant's claimed invention, given the teachings of Knudsen and Kornfelt.

For at least these reasons, this ground for rejection should be withdrawn.

B. Rejection Of Claims 11-12 And 74-75 As Being Obvious Over Knudsen In View Of Kornfelt And Further In View Of Hora

Claims 11-12 and 74-75 were rejected under 35 U.S.C. § 103(a) as being obvious over Knudsen in view of Kornfelt and further in view of Hora et al. (U.S. Patent No. 5,997,856, "Hora"). Applicant respectfully traverses the rejection.

As discussed above, the Examiner has failed to establish a *prima facie* case of obviousness for the rejection of the claims over Knudsen in view of Kornfelt. Hora does not remedy the deficiencies of Knudsen and Kornfelt. Hora discloses a method for the solubilization and/or stabilization of polypeptides using cyclodextrin. Hora fails to disclose the presently claimed combination of GLP-2, histidine, phosphate buffer, and a bulking agent. Accordingly, claims 11-12 and 74-75 are not obvious over Knudsen in view of Kornfelt and further in view of Hora. Applicant respectfully requests withdrawal of the rejection.

C. Rejection Of Claims 13-15, 17-20 And 76 As Being Obvious Over Knudsen In View Of Kornfelt And Further In View Of Drucker (WO 97/39031, "Drucker A")

Claims 13-15, 17-20 and 76 were rejected under 35 U.S.C. § 103(a) as being obvious over Knudsen in view of Kornfelt and further in view of Drucker et al. (WO 97/39031, "Drucker A"). Applicant respectfully traverses the rejection.

As discussed above, the Examiner has failed to establish a *prima facie* case of obviousness for the rejection of the claims over Knudsen in view of Kornfelt. Drucker A does not remedy the deficiencies of Knudsen and Kornfelt. Drucker A discloses analogs of GLP-2, formulations comprising the analogs, and uses thereof. However, Drucker A fails to disclose the presently claimed combination of GLP-2, histidine, phosphate buffer, and a bulking agent. Therefore, claims 13-15, 17-20 and 76 are not obvious over Knudsen in view of Kornfelt and further in view of Drucker A. Applicant respectfully requests withdrawal of the rejection.

D. Rejection Of Claims 16 And 21 As Being Obvious Over Knudsen In View Of Kornfelt And Further In View Of Thim

Claims 16 and 21 were rejected under 35 U.S.C. § 103(a) as being obvious over Knudsen in view of Kornfelt and further in view of Thim et al. (U.S. Patent No. 5,912,229, "Thim"). Applicant respectfully traverses and requests withdrawal of the rejection.

As discussed above, the Examiner has failed to establish a *prima facie* case of obviousness for the rejection of the claims over Knudsen in view of Kornfelt. Thim does not remedy the deficiencies of Knudsen and Kornfelt. Thim relates to use of a pharmaceutical composition comprising GLP-2 or an analog thereof. However, Thim fails to disclose the presently claimed combination of GLP-2, histidine, phosphate buffer, and a bulking agent. Therefore, claims 16 and 21 are not obvious over Knudsen in view of Kornfelt and further in view of Thim. Applicant respectfully requests withdrawal of the rejection.

E. Rejection Of Claims 43-46 And 77 As Being Obvious Over Knudsen In View Of Kornfelt And Further In View Of Drucker (U.S. Patent No. 5,952,301, "Drucker B")

Claims 43-46 and 77 were rejected under 35 U.S.C. § 103(a) as being obvious over Knudsen in view of Kornfelt and further in view of Drucker et al. (U.S. Patent No. 5,952,301, "Drucker B"). Applicant respectfully traverses and requests withdrawal of the rejection.

As discussed above, the Examiner has failed to establish a *prima facie* case of obviousness for the rejection of the claims over Knudsen in view of Kornfelt. Drucker B does not remedy the deficiencies of Knudsen and Kornfelt. Drucker B discloses a package having an ampoule containing GLP-2, at least one other peptide hormone, and a carrier, and further incorporating a label instructing the use of its contents. However, Drucker B fails to disclose the presently claimed kit comprising an ampoule having GLP-2, histidine, phosphate buffer and a bulking agent, a second ampoule, and instructions. Accordingly, claims 43-46 and 77 are not obvious over Knudsen in view of Kornfelt and further in view of Drucker B. Applicant respectfully requests withdrawal of the rejection.

IV. Claim Objections

Claims 9, 23-30, 47-48 and 72 were objected to as being dependant upon a rejected base claim. In view of the remarks above, Applicant respectfully requests withdrawal of this ground of objection.

V. Conclusion

The present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date January 4, 2005

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Enclosures:

1. D.J. Drucker, *J. Clin. Endocrinol. Metab. 86(4)*:1759-64 (2001).
2. European Pharmacopoeia Commission, The European Pharmacopeia (2nd Ed.), page 612 (1989).
3. Munroe et al., *Proc. Natl. Acad. Sci. U.S.A. 96(4)*:1569-73 (1999).

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HOT TOPIC

Glucagon-Like Peptide 2*

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ABSTRACT

Glucagon-like peptide 2 (GLP-2) is a 33 amino acid peptide-encoded carboxyterminal to the sequence of GLP-1 in the proglucagon gene. Both GLP-1 and GLP-2 are secreted from gut endocrine cells and promote nutrient absorption through distinct mechanisms of action. GLP-2 regulates gastric motility, gastric acid secretion, intestinal hexose transport, and increases the barrier function of the gut epithelium. GLP-2 significantly enhances the surface area of the mucosal epithelium via stimulation of crypt cell proliferation and inhibition of apoptosis in the enterocyte and crypt compartments. The cytoprotective and reparative effects of GLP-2 are evident in rodent models of experimental intestinal injury. GLP-2 reduces mortality and decreases mucosal injury, cytokine expression, and bacterial septicemia in the setting of small and large bowel inflammation. GLP-2 also enhances nutrient absorption and gut adaptation in rodents or humans with short bowel syndrome. The actions of GLP-2 are transduced by the GLP-2 receptor, a G protein-coupled receptor expressed in gut endocrine cells of the stomach, small bowel, and colon. Activation of GLP-2 receptor signaling in heterologous cells promotes resistance to apoptotic injury *in vitro*. The cytoprotective, reparative, and energy-retentive properties of GLP-2 suggests that GLP-2 may potentially be useful for the treatment of human disorders characterized by injury and/or dysfunction of the intestinal mucosal epithelium. (*J Clin Endocrinol Metab* **86:** 1759–1764, 2001)

THE GLUCAGON-LIKE PEPTIDES (GLPs) are produced in open type gut endocrine cells of the small and large intestine and play diverse roles in the regulation of energy homeostasis. Food ingestion sets in motion coordinated peptidergic responses that regulate nutrient transit through the gut, and nutrient absorption. Gut peptides also contribute to optimization of mucosal epithelial function for nutrient digestion and absorption. Following nutrient absorption into the blood stream, enteroendocrine-derived peptides such as GLP-1 facilitate nutrient disposal by regulating islet hormones and, indirectly, energy absorption via effects on liver, muscle, and adipose tissue. The aim of this review is to highlight recent advances in our understanding of the role played by GLP-2 in the regulation of intestinal epithelial biology and nutrient absorption.

The sequence of proglucagon contains a number of distinct peptides with pleiotropic actions, including glicentin, glucagon, oxyntomodulin, and two GLPs as well as two intervening or spacer peptides (Fig. 1). Whereas glucagon, GLP-1, and GLP-2 exert well-defined actions through known receptors, the biological actions of the remaining proglucagon-derived peptides remain less well characterized. Furthermore, no receptors have yet been isolated for glicentin, oxyntomodulin, or the intervening peptides (1–4).

Although the sequence of GLP-2 was not detected in anglerfish islet proglucagon complementary DNAs (cDNAs; Ref. 5), the GLP-2 sequence (Fig. 2) was detected in all isolated mammalian proglucagon cDNAs and genes (6–10) as a 33 amino acid peptide located carboxyterminal to GLP-1 and intervening peptide 2 (Figs. 1 and 2). Subsequent studies demonstrated that fish, chickens, and lizards generate GLP-2 in the gut as a result of tissue-specific alternative RNA splicing of proglucagon RNA transcripts (11, 12). In mammals, tissue-specific posttranslational processing liberates GLP-2 from proglucagon in the intestine and brain but not in pancreas, as a result of cell-specific expression of prohormone convertases in gut endocrine cells (13). Isolation and sequencing of GLP-2 from the porcine and human intestine confirmed that GLP-2 is a 33 amino acid peptide (Fig 2), corresponding to proglucagon 126–158, ending in a carboxyterminal Asp residue (14, 15).

Initial studies of GLP-2 biological activity demonstrated increased adenylate cyclase activity in rat hypothalamic and pituitary membrane preparations following incubation with 50 pM GLP-2 (16). Subsequent analyses of GLP-2 activity in the gut either failed to demonstrate activity of GLP-2 or demonstrated GLP-2-mediated inhibition of serum-stimulated intestinal cell growth *in vitro* (17). Nevertheless, despite these negative findings, considerable experimental evidence correlated increased expression and secretion of intestinal proglucagon-derived peptides (PGDPs) with bowel injury and mucosal growth of the small intestine (18–22). Furthermore, two patients with glucagonomas exhibited marked small bowel villus hyperplasia that was reversed following surgical removal of the tumor (23, 24). Following observa-

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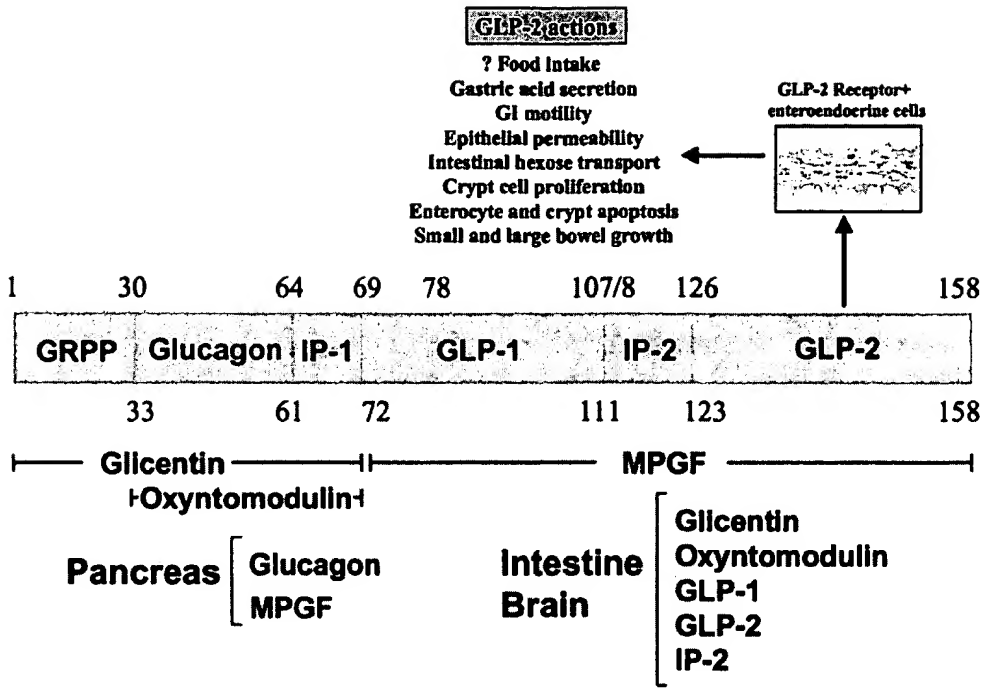

FIG. 1. Structure of mammalian proglucagon, the proglucagon-derived peptides, and the biological actions of GLP-2. The numbers *above* and *below* the proglucagon molecule correspond to the positions and length of specific PGDPs. MPGF, Major proglucagon fragment; IP, intervening peptide.

Glucagon	**HSQGTFTSDYSKYLDSRRAQDFVQWLMNT**
GLP-1	**HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG**
GLP-2	**HADGSFSDEMNTILDNLAARDFINWLIQTKITD**

FIG. 2. Peptide sequences of human glucagon, GLP-1, and GLP-2. Amino acids common to all three peptides are shown in *bold*. Amino acids common to glucagon and GLP-1, GLP-1, and GLP-2, and glucagon and GLP-2 are shown in *green*, *blue*, and *red*, respectively.

tions that mice with sc glucagonomas developed massive small bowel hyperplasia, peptide injection studies identified GLP-2 as the PGDP with significant intestinotrophic activity (25).

GLP-2 synthesis, secretion, and degradation

A single proglucagon messenger RNA (mRNA) transcript, identical in sequence to proglucagon mRNAs in pancreas and brain (Fig. 1), is expressed in the mammalian small and large intestine (26–28). GLP-1 and GLP-2 are cosecreted from the gut (29) with the type and amount of nutrient ingestion representing a primary determinant of both intestinal proglucagon gene expression (30–33) and GLP-2 secretion in rodent, pig, and human studies (34–37). Fiber-enriched diets and fatty acids are potent stimulators of GLP-2 secretion in rodents and human subjects (36, 38).

Intestinal injury or resection is associated with increased levels of proglucagon mRNA transcripts in the intestinal remnant as a result of an increase in proglucagon RNA content in the remaining enteroendocrine cells (39–42). The kidney seems to be an important determinant of GLP-2 clearance as levels of immunoreactive GLP-2 are increased in human patients with renal failure (43). Similarly, experimental nephrectomy results in delayed clearance and increased circulating levels of GLP-2 in rats (44, 45). The estimated elimination $t_{1/2}$ of exogenously administered GLP-2 in human studies seems to be ~7.2 min (46), considerably longer than the $t_{1/2}$ of GLP-1 in similar studies.

Analysis of rat and human plasma using a combination of high-performance liquid chromatography and site-specific GLP-2 antisera reveals the presence of two principal circulating molecular forms, GLP-2^{1-33} and GLP-2^{3-33} (15, 35, 46, 47). GLP-1, gastric inhibitory peptide, and GLP-2 all contain an alanine residue in position 2, rendering them ideal substrates for degradation by dipeptidyl peptidase IV (DP IV), a ubiquitous protease expressed in the gut and vascular endothelium (48, 49). Incubation of GLP-2 with DP IV *in vitro* results in cleavage to the bioinactive GLP-2^{3-33} peptide, and inhibitors of DP IV prevent GLP-2 degradation both *in vitro* and *in vivo* (35, 46, 47). The importance of DP IV for the biological activity of GLP-2 is exemplified by studies in rats demonstrating considerably greater intestinotrophic activity of an exogenously administered GLP-2 analog resistant to DP IV-mediated inactivation (47). Similarly coadministration of a DP IV inhibitor potentiates the trophic activity of exogenous native GLP-2 in rats (50).

Increased circulating levels of GLP-2 are associated with the development of intestinal mucosal hyperplasia in rodents with experimental diabetes (38, 51). Administration of insulin to diabetic rats reduces the levels of circulating GLP-2 and reverses the small bowel mucosal hyperplasia (51). Human subjects with inflammatory bowel disease exhibit normal to increased levels of circulating bioactive GLP-2^{1-33} (52), at-

tributable in part to a decrease in levels of circulating DP IV activity. Patients with Crohn's disease and marked involvement of the terminal ileum and patients with short bowel syndrome and lack of a colon in continuity with the remnant small bowel exhibit deficient levels of circulating GLP-2 and reduced meal-stimulated GLP-2 secretion, respectively (52, 53). In contrast, patients with ileal resection but a preserved colon exhibit increased fasting and meal-stimulated levels of GLP-2 and enhanced intestinal adaptation (54), providing a possible physiological explanation linking the presence of a colon with facilitated intestinal adaptation.

Biological activities of GLP-2

The gastrointestinal tract, from the stomach to the colon, is the principal target for GLP-2 action. GLP-2 inhibits stimulated gastric acid secretion in human subjects and reduces antral gastric motility in the pig (55, 56). Acute GLP-2 infusion rapidly increases intestinal hexose transport in rats, with significant increases in both hexose and SGLT-1 transport activity detectable within 60 min after initiation of iv GLP-2 infusion (57, 58). Administration of GLP-2 to normal mice produces significant increases in intestinal barrier function. Reduced epithelial permeability as measured by decreased ion and macromolecule transport, is detectable in Ussing chamber studies after a single injection of GLP-2 (59). Morphologically, intestinal epithelial cells appear narrower and longer, with increased numbers of longer microvilli detectable on the luminal surface of the enterocyte following several days of GLP-2 administration (59).

GLP-2 administered exogenously to mice and rats promotes expansion of the mucosal epithelium in the small and large bowel, with the most prominent trophic effects seen in the small bowel, specifically in the jejunum (25, 60). The trophic effects of GLP-2 are independent of the route of GLP-2 administration and are observed after iv, sc, or ip GLP-2 administration (57, 60–62). Although the optimal dosing and timing of GLP-2 administration for human clinical studies remains to be determined, GLP-2 is intestinotrophic in rodents even in daily or every other day administration regimens (60, 61). Whereas small changes in intestinal length have been detected after GLP-2 treatment (63), increased thickness of the intestinal mucosa, predominantly an increase in small bowel villus height and mucosal surface area, is invariably detected after several days of GLP-2 treatment (25, 47, 60, 61, 63). Enhanced thickness of the mucosal epithelium may be explained by GLP-2-stimulated increases in crypt cell proliferation, coupled with a decrease in the rate of enterocyte apoptosis (25, 61).

The GLP-2-treated bowel is functionally normal as assessed by analysis of macromolecule expression, and normal levels of mucosal enzymes are observed in the GLP-2-treated murine intestine (64). Absorption of carbohydrates, lipids, and proteins is normal to enhanced in GLP-2-treated mice (64). Similarly, GLP-2-treated rats exhibit enhanced absorption of glycine and galactose in association with increased mucosal DNA and protein content (65). Despite the suggestion that intracrebroventricular GLP-2 administration inhibits food intake (66), GLP-2 treatment of normal animals results in normal food intake, weight gain commensurate with intestinal growth and an enhanced capacity for nutrient absorption (64).

The finding that enteral nutrients regulate GLP-2 secretion suggests a role for GLP-2 in mediating the trophic effect of nutrients on maintaining the normal thickness of the mucosal epithelium. Rats maintained on parenteral nutrition develop atrophy of the intestinal epithelium in both the small and large intestine, possibly as a result of reduced GLP-2 secretion. Intravenous coinfusion of GLP-2 and parenteral nutrition prevented the development of mucosal atrophy in the small but not the large bowel, illustrating the differential regional sensitivity of the gut to the trophic effects of GLP-2 (62, 67). Consistent with these findings, GLP-2 significantly improved the endogenous intestinal adaptive response to major small bowel resection in rats, with increased nutrient absorption and reduced intestinal permeability observed in the GLP-2-treated animals (68, 69).

The trophic and reparative effects of GLP-2 on the gut mucosa have also been observed in the setting of experimental intestinal injury. Following induction of indomethacin-induced intestinal inflammation, GLP-2 significantly reduced intestinal disease activity scores and cytokine expression, decreased bacterial sepsis, and reduced mortality in mice with enteritis (70). Remarkably, GLP-2 was most effective in ameliorating disease activity when administered as a pretreatment regimen before onset of indomethacin-induced enteritis (70). GLP-2 also increased mucosal DNA content and significantly reduced mortality in rats following vascular ischemia-reperfusion injury of the small intestine (71). The protective effects of GLP-2 in the gut have also been observed in the large bowel as mice with dextran sulfate colitis exhibit reduced parameters of disease activity, decreased intestinal interleukin expression, and significantly reduced weight loss after GLP-2 administration (72). Similarly, GLP-2 significantly reduced gross and microscopic mucosal damage and decreased cytokine expression in rats with antigen-induced inflammatory bowel disease (73).

The detection of GLP-2 receptor mRNA transcripts in the fetal and neonatal rat intestine (74) raises the possibility that GLP-2 may play a role in the development and maturation of the gastrointestinal tract. Daily administration of h[Gly2]-GLP-2 to neonatal rats enhanced stomach and small bowel weight and small bowel length (74). Furthermore, iv infusion of GLP-2 decreased proteolysis, reduced apoptosis, increased villus height, and was trophic to the gastrointestinal tract of immature pigs (75). Whether GLP-2 plays a role in growth and differentiation of the developing fetal gut remains unclear.

Mechanisms underlying GLP-2 action: the GLP-2 receptor

The actions of GLP-2 in the gut are mediated by a distinct GLP-2 receptor, a recently cloned member of the glucagon/GLP-1 G protein-coupled receptor superfamily (76). GLP-2R cDNAs isolated from intestinal and hypothalamic cDNA libraries are identical in sequence and encode a predicted receptor of 550 amino acids, exhibiting considerable amino acid identity with the glucagon and GLP-1 receptors. The GLP-2R gene was localized to human chromosome 17p13.3, a chromosomal region not yet linked to inheritance of fa-

milial intestinal diseases. Activation of GLP-2R signaling is coupled to an increase in cAMP with an EC_{50} of ~0.58 nM GLP-2. In contrast, structurally related peptides such as glucagon, GLP-1, GIP, or exendin-4 do not activate the GLP-2R even at 10-nM concentrations (76). The intestinotrophic properties of GLP-2 derivatives in mice *in vivo* correlate well with the relative activation of GLP-2R signaling in transfected fibroblasts by these same peptides *in vitro* (76, 77). Analysis of the activity of alanine-substituted and both N- and C-terminally deleted GLP-2 molecules using the transfected GLP-2R expressed in fibroblasts identified a number of amino acid substitutions in the GLP-2 molecule that result in either diminished receptor binding and/or reduced receptor activation *in vitro* (77).

The GLP-2R is expressed in a highly tissue-specific manner, predominantly in the stomach, jejunum, ileum, and colon (76, 78). The results of Northern blotting, RNase protection, and RT-PCR experiments are consistent with the presence of a single GLP-2R transcript in the gastrointestinal tract and central nervous system of rodents and humans (78). The GLP-2R has been localized to subsets of enteroendocrine cells in the human gut. GLP-2R+ gut endocrine cells also exhibit immunopositivity for either GIP, serotonin, peptide YY, chromogranin, or GLP-1 (78). Although GLP-2 presumably exerts direct effects on enteroendocrine cells expressing the GLP-2R, it seems likely that many of the effects of GLP-2 on gastrointestinal target cells that do not express the GLP-2R are indirect, resulting in modulation of gastric motility, small bowel permeability, and both crypt cell proliferation and apoptosis. Hence, one model that explains GLP-2 action suggests that GLP-2 synthesized in and secreted from the small and large intestine exerts many of its actions in an autocrine, paracrine, or endocrine manner by stimulating the release of as yet unidentified mediators from GLP-2R+ gut endocrine cells. It seems likely that these GLP-2R+ enteroendocrine cells then release one or more factors that mediate the pleiotropic biological actions of GLP-2 in the gut (Fig. 1).

The observations that GLP-2 inhibits enterocyte and crypt compartment apoptosis following intestinal injury (70) prompted analysis of the mechanisms coupling GLP-2R signaling to reduced cell death. Remarkably, direct activation of GLP-2R signaling in transfected baby hamster kidney fibroblasts expressing the GLP-2 receptor (BHK-GLP-2R cells) confers resistance to cycloheximide-induced apoptosis (79). GLP-2 reduced activation of caspase-8, caspase-9, decreased cytochrome c release, and reduced caspase-3 cleavage, in a protein kinase A-independent manner. The antiapoptotic actions of GLP-2 are not diminished by inhibitors of the phosphatidyl inositol-3 kinase or mitogen-activated protein kinase pathways (79). Furthermore, GLP-2 enhanced survival and decreased intestinal apoptosis in tumor-bearing mice treated with chemotherapy and reduced apoptosis and caspase activation in BHK-GLP-2R cells treated with irinotecan *in vitro* (80). These findings demonstrating a direct antiapoptotic effect of GLP-2 on cells expressing a GLP-2 receptor, taken together with the cytoprotective effects of GLP-2 *in vivo* on target cells that do not seem to express the GLP-2 receptor (70, 72, 80) suggest that GLP-2 inhibits cell death via both direct and indirect signaling pathways.

Summary of current knowledge and unanswered questions

The available data demonstrate that GLP-2 regulates motility, nutrient absorption, epithelial permeability, cell proliferation, and apoptosis in the gastrointestinal tract. Whether one or more of these actions will prove to be essential for normal gut physiology in the absence of intestinal injury awaits the development of GLP-2 antagonists or rodent models of disrupted GLP-2 action. Similarly, the actions of GLP-2 have been principally delineated in rodents and the biological activities of GLP-2 in human subjects currently remain unclear. Nevertheless, the strong conservation of GLP-2 and GLP-2R sequences across species suggests that the physiological actions of GLP-2 in rodents and humans are likely to be comparable. In this regard, the results of a recent study of GLP-2 administration in human subjects with short bowel syndrome demonstrated enhanced energy absorption and increased crypt plus villus height in GLP-2-treated patients (81). Given the expression of the GLP-2 receptor in the central nervous system (76, 78), it seems likely that GLP-2, like GLP-1, also subserves one or more functions in the brain. Indeed, intracerebroventricular injection of GLP-2 in the rat reduces food intake (66), raising the possibility that GLP-2, perhaps like GLP-1, acts as a central satiety factor. The multiple actions of GLP-2 that include protection and restoration of the gut epithelium and enhancement of nutrient absorption will likely stimulate clinical testing of the therapeutic potential of this peptide in human diseases characterized by injury and/or dysfunction of the gut epithelium. Whether GLP-2 will ultimately prove therapeutically useful and safe for the treatment of human gastrointestinal diseases requires careful assessment in properly controlled clinical trials.

References

1. **Drucker DJ.** 1998 The glucagon-like peptides. Diabetes. 47:159–169.
2. **Drucker DJ.** 2001 The glucagon-like peptides. Endocrinology. 42:521–527.
3. **Holst JJ.** 1997 Enteroglucagon. Annu Rev Physiol. 59:257–291.
4. **Kieffer TJ, Habener JF.** 1999 The glucagon-like peptides. Endocr Rev. 20:876–913.
5. **Lund PK, Goodman RH, Dee PC, Habener JF.** 1982 Pancreatic preproglucagon cDNA contains two glucagon-related coding sequences arranged in tandem. Proc Natl Acad Sci USA. 79:345–349.
6. **Bell GI, Santerre RF, Mullenbach GT.** 1983 Hamster preproglucagon contains the sequence of glucagon and two related peptides. Nature. 302:716–718.
7. **Bell GI, Sanchez-Pescador R, Laybourn PJ, Najarian RC.** 1983 Exon duplication and divergence in the human preproglucagon gene. Nature. 304:368–371.
8. **Heinrich G, Gros P, Habener JF.** 1984 Glucagon gene sequence: four of the six exons encode separate functional domains of rat pre-proglucagon. J Biol Chem. 259:14082–14087.
9. **Heinrich G, Gros P, Lund PK, Bentley RC, Habener JF.** 1984 Pre-proglucagon messenger ribonucleic acid: nucleotide and encoded amino acid sequences of the rat pancreatic complementary deoxyribonucleic acid. Endocrinology. 115:2176–2181.
10. **White JW, Saunders GF.** 1986 Structure of the human glucagon gene. Nucleic Acids Res. 14:4719–4730.
11. **Irwin DM, Wong J.** 1995 Trout and chicken proglucagon: alternative splicing generates mRNA transcripts encoding glucagon-like peptide 2. Mol Endocrinol. 9:267–277.
12. **Chen YE, Drucker DJ.** 1997 Tissue-specific expression of unique mRNAs that encode proglucagon-derived peptides or exendin 4 in the lizard. J Biol Chem. 272:4108–4115.
13. **Dhanvantari S, Seidah NG, Brubaker PL.** 1996 Role of prohormone convertases in the tissue-specific processing of proglucagon. Mol Endocrinol. 10:342–355.
14. **Orskov C, Buhl T, Rabenhoj L, Kofod H, Holst JJ.** 1989 Carboxypeptidase-B-like processing of the C-terminus of glucagon-like peptide-2 in pig and human small intestine. FEBS Lett. 247:193–196.
15. **Hartmann B, Johnsen AH, Orskov C, Adelhorst K, Thim L, Holst JJ.** 2000

Structure, measurement, and secretion of human glucagon-like peptide-2. Peptides. 21:73–80.
16. **Hoosein NM, Gurd RS.** 1984 Human glucagon-like peptides 1 and 2 activate rat brain adenylate cyclase. FEBS Lett. 178:83–86.
17. **Lund PK, Ulshen MH, Roundtree DB, Selub SE, Buchan AM.** 1990 Molecular biology of gastrointestinal peptides and growth factors: relevance to intestinal adaptation. Digestion. 46:66–73.
18. **Bloom SR, Polak JM.** 1982 The hormonal pattern of intestinal adaptation [a major role for enteroglucagon]. Scand J Gastroenterol. 17:93–103.
19. **Besterman HS, Adrian TE, Mallinson CN, et al.** 1982 Gut hormone release after intestinal resection. Gut. 23:854–861.
20. **Sagor GR, Ghatei MA, Al-Mukhtar MYT, Wright NA, Bloom SR.** 1983 Evidence for a humoral mechanism after small intestinal resection. Gastroenterology. 84:902–906.
21. **Miazza BM, Al-Mukhtar MYT, Salmeron M, et al.** 1985 Hyperenteroglucagonaemia and small intestinal mucosal growth after colonic perfusion of glucose in rats. Gut. 26:518–524.
22. **Taylor RG, Fuller PJ.** 1994 Humoral regulation of intestinal adaptation. Baillieres Clin Endocrinol Metab. 8:165–183.
23. **Gleeson MH, Bloom SR, Polak JM, Henry K, Dowling RH.** 1971 Endocrine tumour in kidney affecting small bowel structure, motility, and absorptive function. Gut. 12:773–782.
24. **Stevens FM, Flanagan RW, O'Gorman D, Buchanan KD.** 1984 Glucagonoma syndrome demonstrating giant duodenal villi. Gut. 25:784–791.
25. **Drucker DJ, Ehrlich P, Asa SL, Brubaker PL.** 1996 Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proc Natl Acad Sci USA. 93:7911–7916.
26. **Drucker DJ, Asa S.** 1988 Glucagon gene expression in vertebrate brain. J Biol Chem. 263:13475–13478.
27. **Drucker DJ, Brubaker PL.** 1989 Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. Proc Natl Acad Sci USA. 86:3953–3957.
28. **Novak U, Wilks A, Buell G, McEwen S.** 1987 Identical mRNA for preproglucagon in pancreas and gut. Eur J Biochem. 164:553–558.
29. **Orskov C, Holst JJ, Knuhtsen S, Baldissera FGA, Poulsen SS, Nielsen OV.** 1986 Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from pig small intestine but not pancreas. Endocrinology. 119:1467–1475.
30. **Rountree DB, Ulshen MH, Selub S, et al.** 1992 Nutrient-independent increases in proglucagon and ornithine decarboxylase messenger RNAs after jejunoileal resection. Gastroenterology. 103:462–468.
31. **Hoyt EC, Lund PK, Winesett DE, et al.** 1996 Effects of fasting, refeeding and intraluminal triglyceride on proglucagon expression in jejunum and ileum. Diabetes. 45:434–439.
32. **Reimer RA, McBurney MI.** 1996 Dietary fiber modulates intestinal proglucagon messenger ribonucleic acid and postprandial secretion of glucagon-like peptide-1 and insulin in rats. Endocrinology. 137:3948–3956.
33. **Tappenden KA, Thomson AB, Wild GE, McBurney MI.** 1996 Short-chain fatty acids increase proglucagon and ornithine decarboxylase messenger RNAs after intestinal resection in rats. J Parenter Enteral Nutr. 20:357–362.
34. **Orskov C, Holst JJ.** 1987 Radio-immunoassays for glucagon-like peptides 1 and 2 (GLP-1 and GLP-2). Scand J Clin Lab Invest. 47:165–174.
35. **Brubaker PL, Crivici A, Izzo A, Ehrlich P, Tsai C-H, Drucker DJ.** 1997 Circulating and tissue forms of the intestinal growth factor, glucagon-like peptide 2. Endocrinology. 138:4837–4843.
36. **Xiao Q, Boushey RP, Drucker DJ, Brubaker PL.** 1999 Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. Gastroenterology. 117:99–105.
37. **Burrin DG, Stoll B, Jiang R, et al.** 2000 Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough? Am J Clin Nutr. 71:1603–1610.
38. **Thulesen J, Hartmann B, Nielsen C, Holst JJ, Poulsen SS.** 1999 Diabetic intestinal growth adaptation and glucagon-like peptide 2 in the rat: effects of dietary fibre. Gut. 45:672–678.
39. **Fuller PJ, Beveridge DJ, Taylor RG.** 1993 Ileal proglucagon gene expression in the rat: characterization in intestinal adaptation using *in situ* hybridization. Gastroenterology. 104:459–466.
40. **Taylor RG, Verity K, Fuller PJ.** 1990 Ileal glucagon gene expression: ontogeny and response to massive small bowel resection. Gastroenterology. 99:724–729.
41. **Taylor RG, Beveridge DJ, Fuller PJ.** 1992 Expression of ileal glucagon and peptide tyrosine-tyrosine genes. Response to inhibition of polyamine synthesis in the presence of massive small-bowel resection. Biochem J. 286:737–741.
42. **Ulshen MH, Hoyt EC, Fuller CR, Ghatei MA, Bloom SR, Lund PK.** 1996 Increased ileal proglucagon expression after jejunectomy is not suppressed by inhibition of bowel growth. Dig Dis Sci. 41:677–683.
43. **Orskov C, Andreasen J, Holst JJ.** 1992 All products of proglucagon are elevated in plasma from uremic patients. J Clin Endocrinol Metab. 74:379–384.
44. **Ruiz-Grande C, Pintado J, Alarcon C, Castilla C, Valverde I, Lopez-Novoa JM.** 1990 Renal catabolism of human glucagon-like peptides 1 and 2. Can J Physiol Pharmacol. 68:1568–1573.
45. **Tavares W, Drucker DJ, Brubaker PL.** 1999 Enzymatic and renal-dependent catabolism of the intestinotropic hormone glucagon-like peptide-2 in the rat. Am J Physiol. 278:E134–E139.
46. **Hartmann B, Harr MB, Jeppesen PB, et al.** 2000 *In vivo* and *in vitro* degradation of glucagon-like peptide-2 in humans. J Clin Endocrinol Metab. 85:2884–2888.
47. **Drucker DJ, Shi Q, Crivici A, et al.** 1997 Regulation of the biological activity of glucagon-like peptide 2 by dipeptidyl peptidase IV. Nat Biotechnol. 15:673–677.
48. **Kieffer TJ, McIntosh CHS, Pederson RA.** 1995 Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 *in vitro* and *in vivo* by dipeptidyl peptidase IV. Endocrinology. 136:3585–3596.
49. **De Meester I, Korom S, Van Damme J, Scharpe S.** 1999 CD26, let it cut or cut it down. Immunol Today. 20:367–375.
50. **Hartmann B, Thulesen J, Kissow H, et al.** 2000 Dipeptidyl peptidase IV inhibition enhances the intestinotrophic effect of glucagon-like peptide-2 in rats and mice. Endocrinology. 141:4013–4020.
51. **Fischer KD, Dhanvantari S, Drucker DJ, Brubaker PL.** 1997 Intestinal growth is associated with elevated levels of glucagon-like peptide-2 in diabetic rats. Am J Physiol. 273:E815–E820.
52. **Xiao Q, Boushey RP, Cino M, Drucker DJ, Brubaker PL.** 2000 Circulating levels of glucagon-like peptide-2 in human subjects with inflammatory bowel disease. Am J Physiol. 278:R1057–R1063.
53. **Jeppesen PB, Hartmann B, Hansen BS, Thulesen J, Holst JJ, Mortensen PB.** 1999 Impaired meal-stimulated glucagon-like peptide-2 response in ileal resected short bowel patients with intestinal failure. Gut. 45:559–563.
54. **Jeppesen PB, Hartmann B, Thulesen J, et al.** 2000 Elevated plasma glucagon-like peptide 1 and 2 concentrations in ileum resected short bowel patients with a preserved colon. Gut. 47:370–376.
55. **Wojdemann M, Wettergren A, Hartmann B, Hilsted L, Holst JJ.** 1999 Inhibition of sham feeding-stimulated human gastric acid secretion by glucagon-like peptide-2. J Clin Endocrinol Metab. 84:2513–2517.
56. **Wojdemann M, Wettergren A, Hartmann B, Holst JJ.** 1998 Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. Scand J Gastroenterol. 33:828–832.
57. **Cheeseman CI, Tsang R.** 1996 The effect of gastric inhibitory polypeptide and glucagon like peptides on intestinal hexose transport. Am J Physiol Gastrointest Liver Physiol. 271:G477–G482.
58. **Cheeseman CI.** 1997 Up-regulation of SGLT-1 transport activity in rat jejunum induced by GLP-2 infusion *in vivo*. Am J Physiol. 273:R1965–R1971.
59. **Benjamin MA, McKay DM, Yang P-C, Perdue MH.** 2000 Glucagon-like peptide-2 enhances epithelial barrier function of both transcellular and paracellular pathways in the mouse. Gut. 47:112–119.
60. **Tsai C-H, Hill M, Asa SL, Brubaker PL, Drucker DJ.** 1997 Intestinal growth-promoting properties of glucagon-like peptide 2 in mice. Am J Physiol. 273:E77–E84.
61. **Tsai C-H, Hill M, Drucker DJ.** 1997 Biological determinants of intestinotrophic properties of GLP-2 *in vivo*. Am J Physiol. 272:G662–G668.
62. **Chance WT, Foley-Nelson T, Thomas I, Balasubramaniam A.** 1997 Prevention of parenteral nutrition-induced gut hyoplasia by coinfusion of glucagon-like peptide-2. Am J Physiol. 273:G559–G563.
63. **Drucker DJ, Deforest L, Brubaker PL.** 1997 Intestinal response to growth factors administered alone or in combination with h[Gly2]-glucagon-like peptide 2. Am J Physiol. 273:G1252–G1262.
64. **Brubaker PL, Izzo A, Hill M, Drucker DJ.** 1997 Intestinal function in mice with small bowel growth induced by glucagon-like peptide-2. Am J Physiol. 272:E1050–E1058.
65. **Kato Y, Yu D, Schwartz MZ.** 1999 Glucagon-like peptide-2 enhances small intestinal absorptive function and mucosal mass *in vivo*. J Pediatr Surg. 34:18–20.
66. **Tang-Christensen M, Larsen PJ, Thulesen J, Romer J, Vrang N.** 2000 The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. Nat Med. 6:802–807.
67. **Kitchen PA, Fitzgerald AJ, Goodlad RA, et al.** 2000 Glucagon-like peptide-2 increases sucrase-isomaltase but not caudal- related homeobox protein-2 gene expression. Am J Physiol Gastrointest Liver Physiol. 278:G425–G428.
68. **Scott RB, Kirk D, MacNaughton WK, Meddings JB.** 1998 GLP-2 augments the adaptive response to massive intestinal resection in rat. Am J Physiol. 275:G911–G921.
69. **Sigalet DL, Martin GR.** 2000 Hormonal therapy for short bowel syndrome. J Pediatr Surg. 35:360–363; discussion, 364.
70. **Boushey RP, Yusta B, Drucker DJ.** 1999 Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. Am J Physiol. 277:E937–E947.
71. **Prasad R, Alavi K, Schwartz MZ.** 2000 Glucagon-like peptide-2 analogue enhances intestinal mucosal mass after ischemia and reperfusion. J Pediatr Surg. 35:357–359.
72. **Drucker DJ, Yusta B, Boushey RP, Deforest L, Brubaker PL.** 1999 Human [Gly2]-GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. Am J Physiol. 276:G79–G91.
73. **Alavi K, Schwartz MZ, Palazzo JP, Prasad R.** 2000 Treatment of inflammatory bowel disease in a rodent model with the intestinal growth factor glucagon-like peptide-2. J Pediatr Surg. 35:847–851.
74. **Lovshin J, Yusta B, Iliopoulos I, et al.** 2000 Ontogeny of the glucagon-like

peptide-2 receptor axis in the developing rat intestine. Endocrinology. 141:4194–4201.
75. **Burrin DG, Stoll B, Jiang R, et al.** 2000 GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis. Am J Physiol Gastrointest Liver Physiol. 279:G1249–G1256.
76. **Munroe DG, Gupta AK, Kooshesh P, et al.** 1999 Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. Proc Natl Acad Sci USA. 96:1569–1573.
77. **DaCambra MP, Yusta B, Sumner-Smith M, Crivici A, Drucker DJ, Brubaker PL.** 2000 Structural determinants for activity of glucagon-like peptide-2. Biochemistry. 39:8888–8894.
78. **Yusta B, Huang L, Munroe D, et al.** 2000 Enteroendocrine localization of GLP-2 receptor expression. Gastroenterology. 119:744–755.
79. **Yusta B, Boushey RP, Drucker DJ.** 2000 The glucagon-like peptide-2 receptor mediates direct inhibition of cellular apoptosis via a cAMP-dependent protein kinase-independent pathway. J Biol Chem. 275:35345–35352.
80. **Boushey RP, Yusta B, Drucker DJ.** 2001 Glucagon-like peptide-2 (GLP-2) reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor. Cancer Res. 61:687–693.
81. **Jeppesen PB, Hartmann B, Thulesen J, et al.** Treatment of short bowel patients with glucagon-like peptide-2 (GLP-2), a newly discovered intestinotrophic, anti-secretory, and transit-modulating peptide. Gastroenterology. In press.

GLUCAGONUM

Glucagon

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-
Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr

$C_{153}H_{225}N_{43}O_{49}S$ M_r 3482

Glucagon is a polypeptide hormone obtained from beef or pork pancreas and which increases the blood-glucose concentration by promoting rapid breakdown of liver glycogen. The potency is not less than 1 I.U. per milligram, calculated with reference to the dried substance. Glucagon is prepared in conditions designed to minimise microbial contamination.

CHARACTERS

A white or almost white powder, practically insoluble in water and in most organic solvents. It dissolves in dilute mineral acids and in dilute solutions of the alkali hydroxides.

IDENTIFICATION

A. It causes a rise of blood-glucose concentration in the test animals when injected as prescribed in the Assay.

B. Examine the electrophoretograms obtained in the test for related substances. The principal band in the electrophoretogram obtained with test solution (a) corresponds in position to the principal band in the electrophoretogram obtained with reference solution (a).

TESTS

Absorbance Dissolve 2.5 mg in 0.01N hydrochloric acid and dilute to 10.0 ml with the same acid. The specific absorbance (V.6.19) determined at the maximum at 276 nm is 21 to 25, calculated with reference to the dried substance.

Related substances Examine by polyacrylamide gel electrophoresis (V.6.21), using rod gels 75 mm long and 5 mm in diameter and, as buffer, tris-glycine buffer solution pH 8.3 R. The electrode in the upper reservoir is the cathode and that in the lower reservoir the anode.

Use the following gel mixture: mix 1 volume of a solution containing in 100 ml 36.6 mg of tris(hydroxymethyl)aminomethane R, 0.23 ml of tetramethylethylenediamine R and 48.0 ml of 1N hydrochloric acid and 2 volumes of a solution containing in 100 ml 0.735 g of methylenebisacrylamide R and 30.0 g of acrylamide R. Add sufficient urea R to give a concentration of 48.0 per cent *m/V* in the final solution and dilute to 7 volumes with water. If necessary, heat to not more than 40 °C to dissolve the urea. Degas the solution and add 1 volume of a 0.56 per cent *m/V* solution of ammonium persulphate R.

Test solution (a) Dissolve 10 mg of the substance to be examined in 0.5 ml of 0.01N sodium hydroxide.

Test solution (b) Dilute 0.25 ml of test solution (a) to 5 ml with 0.01N sodium hydroxide.

Reference solution (a) Dissolve a quantity of glucagon BRP equivalent to 5 I.U. in 0.01N sodium hydroxide and dilute to 25 ml with the same solvent.

Reference solution (b) Dilute 8 ml of reference solution (a) to 10 ml with 0.01N sodium hydroxide.

Reference solution (c) Dilute 6 ml of reference solution (a) to 10 ml with 0.01N sodium hydroxide.

Reference solution (d) Dilute 4 ml of reference solution (a) to 10 ml with 0.01N sodium hydroxide.

Reference solution (e) Dilute 2 ml of reference solution (a) to 10 ml with 0.01N sodium hydroxide.

Apply 100 µl of each solution separately to the surface of a gel. After the addition of the buffer, add 0.2 ml of bromophenol blue solution R. Allow electrophoresis to take place with a constant current of 1 mA per tube for 30 min and then increase the current to 3 mA per tube. Immerse the gels in a 12.5 per cent *m/V* solution of trichloroacetic acid R for at least 1 h. For each 10 ml of trichloroacetic acid solution used add 0.5 ml of a 0.25 per cent *m/V* solution of acid blue 90 R and allow to stand for 12 h. Remove the staining solution and wash twice with a mixture of 1 volume of acetic acid R and 4 volumes of water, discard the washings and store the gels in a similar mixture of acetic acid R and water. Examine the electrophoretograms using a cold-light illuminator. In the electrophoretograms obtained with test solution (a), any band, other than the bromophenol blue band, that migrates

between two and three times the distance of the principal band is not more intense than the principal band in the electrophoretogram obtained with reference solution (e). In the electrophoretogram obtained with test solution (b), any band that migrates immediately in front of the principal band is not more intense than the principal band in the electrophoretogram obtained with reference solution (a). The test is not valid unless a band is seen in the electrophoretogram obtained with reference solution (e) and a gradation of intensity of staining is seen in the electrophoretograms obtained with reference solutions (a) to (e).

Nitrogen 16.0 per cent to 18.5 per cent, determined by the method of sulphuric acid digestion (V.5.5.2) and calculated with reference to the dried substance.

Zinc Not more than 0.15 per cent of Zn, determined by atomic absorption spectrophotometry (Method I, V.6.17).

Test solution Dissolve 50.0 mg of the substance to be examined in 0.01N hydrochloric acid and dilute to 25.0 ml with the same acid. Dilute if necessary with 0.01N hydrochloric acid to obtain a zinc concentration of 0.4 µg to 1.6 µg per millilitre.

Reference solutions Use solutions containing 0.10 µg, 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting zinc standard solution (5 mg/ml Zn) R with 0.01N hydrochloric acid.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as the source of radiation and an air-acetylene flame of suitable composition (for example, 2 litres of acetylene and 11 litres of air per minute).

Loss on drying (V.6.22) Not more than 10.0 per cent, determined on 50.0 mg by drying at 105 °C for 24 h.

ASSAY

The potency of glucagon is determined by comparing, in given conditions, the hyperglycaemic effect it produces with that produced by the International Standard or by a reference preparation calibrated in International Units.

The International Unit is the specific hyperglycaemic activity contained in a stated amount of the International Standard which consists of a quantity of freeze-dried glucagon to which lactose and sodium chloride have been added.[1]

(1) The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The fiducial limits or error (P = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Reference solution Dissolve the entire contents of an ampoule of the reference preparation in 2 ml of a 0.9 per cent *m/V* solution of sodium chloride R adjusted to pH 3.0 with hydrochloric acid R and dilute with the same solvent to obtain a solution containing, for example, 0.1 I.U. per millilitre. Store the solution at 2 °C to 8 °C and use within 2 days.

Use in the test healthy rabbits, the difference in mass between the heaviest and the lightest being not greater than 1.2 kg (for example, rabbits with body masses in the range 1.8 kg to 2.8 kg). Keep the rabbits in the laboratory under uniform conditions on a uniform diet for a least 1 week before use in the assay. Handle the rabbits with care to avoid undue excitement.

Inject intramuscularly into each rabbit 48 h before the test 1 ml of a 2.5 per cent *m/V* solution of cortisone acetate and withhold all food but not water from 16 h before each test day until after the withdrawal of the last blood sample on that day. Distribute the rabbits at random into four equal groups of not fewer than four rabbits.

Prepare two dilutions of the reference solution with a concentration ratio of 1 to 4, for example 0.006 I.U. per millilitre (reference dilution 1) and 0.024 I.U. per millilitre (reference dilution 2). Use as diluent a 0.9 per cent *m/V* solution of sodium chloride R adjusted to pH 3.0 with hydrochloric acid R. Use the same diluent to dissolve the substance to be examined and to prepare two dilutions, one of which (test dilution 1) is presumed, on the basis of the stated potency to contain the same number of units per millilitre as reference dilution 1 and the other (test dilution 2) the same number of units per millilitre as reference dilution 2. Inject 1.0 ml of each dilution subcutaneously giving the doses in the order indicated in the following table, the second injection being made approximately 24 h after the first injection.

	Group of rabbits			
	1	2	3	4
First injection	reference dilution 1	reference dilution 2	test dilution 1	test dilution 2
Second injection	test dilution 2	test dilution 1	reference dilution 2	reference dilution 1

Take a suitable blood sample from the marginal ear vein of each rabbit 1 h after each injection[1] and determine the blood-glucose concentration in each sample.

Calculate the potency by the usual statistical methods for the twin cross-over assay.

Glucagon intended for use in the manufacture of a parenteral dosage form without a further appropriate sterilisation procedure complies with the following additional requirement:

Sterility (V.2.1.1)

STORAGE

Store in an airtight container at a temperature below 8 °C and preferably at − 20 °C.

LABELLING

The labelling complies with the relevant national legislation and international agreements.

The label on the *container* includes a statement of:

— the number of units per container,

— the number of units per milligram,

— the storage conditions.

The label on the *container* and the label on the *package* include a statement, where appropriate, that the substance is sterile.

(1) The optimal time depends on the strain; it is important that for a given assay a definite and exactly identical time interval be scrupulously respected for each rabbit.

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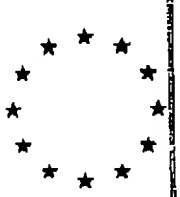

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Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2

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ABSTRACT **Glucagon-like peptide 2 (GLP-2) is a 33-aa proglucagon-derived peptide produced by intestinal enteroendocrine cells. GLP-2 stimulates intestinal growth and upregulates villus height in the small intestine, concomitant with increased crypt cell proliferation and decreased enterocyte apoptosis. Moreover, GLP-2 prevents intestinal hypoplasia resulting from total parenteral nutrition. However, the mechanism underlying these actions has remained unclear. Here we report the cloning and characterization of cDNAs encoding rat and human GLP-2 receptors (GLP-2R), a G protein-coupled receptor superfamily member expressed in the gut and closely related to the glucagon and GLP-1 receptors. The human GLP-2R gene maps to chromosome 17p13.3. Cells expressing the GLP-2R responded to GLP-2, but not GLP-1 or related peptides, with increased cAMP production (EC_{50} = 0.58 nM) and displayed saturable high-affinity radioligand binding (K_d = 0.57 nM), which could be displaced by synthetic rat GLP-2 (K_i = 0.06 nM). GLP-2 analogs that activated GLP-2R signal transduction *in vitro* displayed intestinotrophic activity *in vivo*. These results strongly suggest that GLP-2, like glucagon and GLP-1, exerts its actions through a distinct and specific novel receptor expressed in its principal target tissue, the gastrointestinal tract.**

Glucagon-like peptides (GLPs) encoded by the proglucagon gene play key roles in glucose homeostasis, gastric emptying, insulin secretion, and appetite regulation (1). Glucagon and GLP-1 exert their effects through distinct G protein-coupled receptors (GPCRs). In contrast, unique receptors for GLP-2, glicentin, and oxyntomodulin have not yet been identified, despite considerable attempts at receptor isolation via classical molecular biology approaches (2). Recent studies have shown that GLP-2 is a potent intestinal growth factor that stimulates crypt cell proliferation and inhibits epithelial apoptosis (3). GLP-2 promotes epithelial proliferation in both small and large intestine; however, the mechanisms utilized by GLP-2 for promotion of intestinal growth remain unclear.

To understand the mechanisms underlying GLP-2 action, we have carried out studies directed at the identification and cloning of the putative GLP-2 receptor. We now have isolated rat and human cDNAs encoding GLP-2-responsive GPCRs, which show highest similarity to receptors for glucagon and GLP-1. The GLP-2R is coupled to activation of adenylate cyclase, and the receptor is expressed selectively in rat hypothalamus and the gastrointestinal tract, known targets of GLP-2 action. These findings establish GLP-2 as a novel hormone that, like glucagon and GLP-1, exerts its actions through a distinct receptor expressed in a highly tissue-restricted manner. The GLP-2R should provide an important target for isolation of small molecules mimicking GLP-2 action and for future studies delineating specific mechanisms underlying GLP-2 action in the gut and central nervous system.

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Methods and Materials

Primers, cDNA Libraries, and Cloning Strategy. Initial attempts at low-stringency hybridization of intestine and brain cDNA libraries using GLP-1R/GlucagonR cDNA sequences were not successful. Two million cDNA clones from rat hypothalamus and rat duodenum/jejunum cDNA libraries subsequently were screened with degenerate oligonucleotides derived from conserved transmembrane II and VII GPCR coding sequences: C4–4 (5′-AACTACATCCACMKGM AYCTGTTYVYGTCBTTCATSCT-3′) (IUB nomenclature) and C9–2R (5′-TCYRNCTGSACCTCMYYRTTGAS-RAARCAGTA-3′) (for nomenclature, see ref. 4). First-round cDNA plugs (1,057) were isolated in this screen. In a complementary strategy, PCR was conducted on intestinal cDNA templates by using sets of degenerate PCR primers, based on conserved transmembrane amino acid motifs from family B GPCRs or from motifs conserved mainly within the glucagon/glucose-dependent insulinotropic polypeptide (GIP)/GLP-1 receptor subfamily. PCR products were Southern-blotted and probed with ^{32}P-end-labeled C4–4 oligonucleotide. PCRs, amplified from rat neonatal intestine cDNA (Stratagene; catalog no. 936508) were chosen for cloning. These products had been amplified with the degenerate primers M2F (5′-TTTTTCTAGAASRTSATSTACACNGT SGGCTAC-3′) (based on conserved transmembrane domain I sequences) and M7R (5′-TTTTCTCGAGCCARCARCCASSWRTART-TGGC-3′) (based on conserved transmembrane III sequences). PCR products were cloned into pBluescript, screened by filter hybridization with the nested C4-4 oligonucleotide, and sequenced, leading to the identification of a sequence fragment from a novel GPCR family B member, designated WBR, that ultimately proved to be the GLP-2R. Two new GLP-2R-specific PCR primers, P23-F1 (5′-TCTGACAGATATGA-CATCCATCCAC-3′) and P23-R1 (5′-TCATCTCCCTCT-TCTTGGCTCTTAC-3′), were used to screen the 1,057 cDNA plugs obtained by hybridization screening, leading to the

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: EBNA, Epstein–Barr nuclear antigen; GPCR, G protein-coupled receptor; GLP-1 and -2, glucagon-like peptide 1 and 2, respectively; GLP-2R, GLP-2 receptor; GIP, glucose-dependent insulinotropic polypeptide.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF105367 and AF105368).

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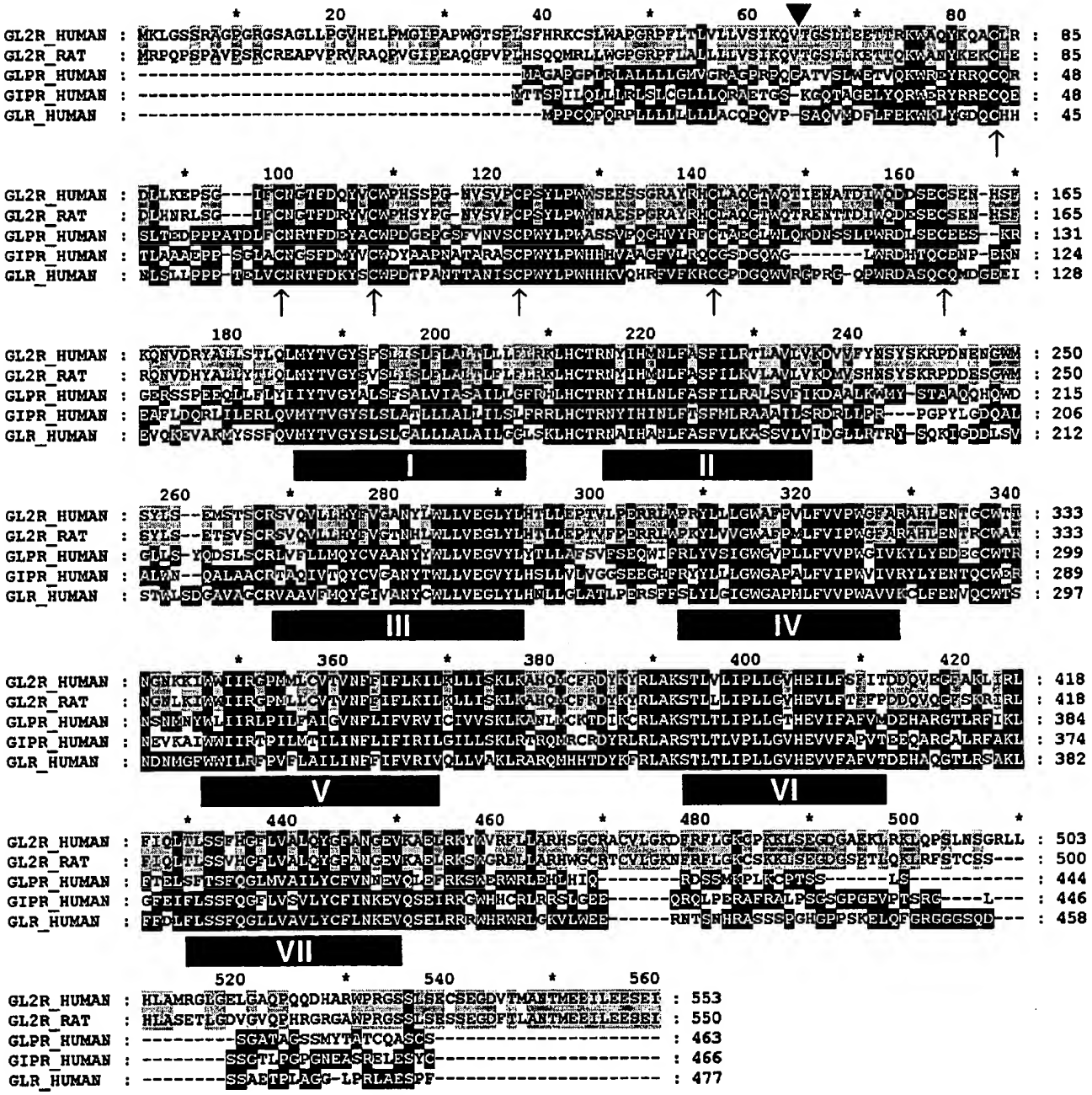

FIG. 1. Multiple alignment of the human (GL2R_HUMAN) and rat (GL2R_RAT) GLP-2R amino acid sequences with human GLP-1, GIP, and glucagon receptor sequences. Known receptor sequences are designated by Swiss-Prot identifiers. Alignment was performed with CLUSTAL W 1.60 and rendered with GENEDOC 2.2. Identities of rat and human GLP-2R sequences is shown in gray, and identities across all five receptor members are indicated by black shading. A predicted signal-peptide cleavage site in human and rat GLP-2R is indicated by an inverted triangle. Six conserved cysteine residues are indicated by arrows. Seven predicted transmembrane domains are shown as solid, black boxes labeled with Roman numerals, and asterisks are shown for spacing every 20 aa. The GenBank accession numbers for the human and rat GLP-2R sequences are AF105367 and AF105368, respectively.

identification of three independent clones, two from the duodenum/jejunum library and a third from hypothalamus, which, together, contained a 2,537-bp cDNA insert encoding full-length rat GLP-2R.

To clone the human GLP-2R, the coding region of the rat GLP-2R was used to screen a human hypothalamus cDNA library (CLONTECH; catalog no. 1172a), from which a single positive clone (HHT13) was isolated and sequenced. A full-length insert was ligated into pcDNA3 for expression studies.

Intestinotrophic Activity, cAMP Determination, and Radioligand-Binding Studies. For cAMP assays, an episomal stable cell line was prepared by lipofection of 293-EBNA (Epstein–Barr nuclear antigen) cells (Invitrogen) with a pREP7-based (Invitrogen) construct containing the Met-42 → Ile-550 ORF of rGLP-2R. Parental 293-EBNA cells, as well as the stable cell line rG2R, expressed receptors for vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide. Therefore, the ligand specificity of GLP-2R was tested further in transiently transfected COS cells, which expressed no functional receptors for any of the ligands tested.

For cAMP assays, cells were treated at 80% confluency with GLP-2 peptide analogs at concentrations ranging from 10^{-12}

to 10^{-5} M for 30 min in medium containing 3-isobutylmethylxanthine. The reaction was terminated with the addition of 95% ethanol and 5 mM EDTA. Aliquots of the ethanol extract were used to determine cAMP levels using an enzyme immunoassay kit (Amersham) as described by the manufacturer. Results were analyzed with GRAPHPAD PRISM software and expressed as pmol cAMP per well. For radioligand-binding assays, cells expressing GLP-2R were harvested and homogenized in 25 mM Hepes (pH 7.4) buffer containing 140 mM NaCl, 0.9 mM $MgCl_2$, 5 mM KCl, 1.8 mM $CaCl_2$, 17 mg/ml Diprotin A, and 100 μM phenanthroline. Homogenates were centrifuged for 10 min at 1,000 $\times$ *g* at 4°C to remove cellular debris. For saturation experiments, membranes containing 25 μg protein were incubated with increasing concentrations of ^{125}I-[Tyr-34]GLP-2 (5–2,000 pM final concentration) in a volume of 0.5 ml for 2 hr at 4°C. Nonspecific binding was determined by the addition of 10 μM of native rat GLP-2 and subtracted from total binding to estimate specific binding to GLP-2R. Parallel experiments confirmed the lack of specific binding when the GLP-2R expression construct was not used. For competition-binding experiments, assays were initiated by the addition of 200 pM (final concentration) of ^{125}I-[Tyr34]GLP-2 with increasing concentrations of competing peptide analogs (10^{-11} to 10^{-5} M) for 2 hr as described above. Reactions were terminated by centrifugation at 13,000 $\times$ *g* for 15 min at 4°C. The pellets were washed three times with cold 50 mM Tris buffer, and radioactivity was quantitated in a gamma counter. Results were analyzed by GRAPHPAD PRISM software.

Intestinotrophic activities of various peptide analogs were determined by assessment of small bowel weight as described (5), after 14-day treatments with 2.5 μg of test peptide or PBS (vehicle-treated control) administered twice daily. Activity was defined as follows: active, small bowel wet weight 40–70% greater than in vehicle-treated control animals; partially active, 20–40% greater than controls; inactive, less than 20% greater than controls.

RNAse Protection Assay. A fragment of GLP-2R cDNA was subcloned into pBluescript (Stratagene) for *in vitro* transcription with T3 or T7 RNA polymerases. The probe, called F1, spanned nucleotides encoding amino acids Met-1 → Arg-210. RNase protection assay was carried out essentially as described (6), using 50 μg of total RNA from adult rat tissues or 50 μg of yeast tRNA (negative control) or tRNA spiked with a known copy number of sense-strand cRNA (for standard curve construction). Each sample was hybridized with 100,000 cpm of [^{32}P]CTP-labeled antisense cRNA and then digested with RNases T1 (140 units/ml) and A (8 μg/ml) at 30 C for 1 hr. The deproteinized, ethanol-precipitated probe was run on a 5% sequencing gel and analyzed after PhosphorImaging (Molecular Dynamics) with IMAGEQUANT software. RNA copy number was calculated by interpolation relative to the standard curve after taking the lengths and specific activities of undigested and digested probes into account. A second RNase probe from a different region of the GLP-2R cDNA was used to confirm the quantitative results (data not shown).

RESULTS AND DISCUSSION

GLP-2 and the peptide hormones GLP-1, glucagon, and GIP have closely related amino acid sequences (7). Similarly, the sequences of cloned receptors for the latter three peptides form a cluster within the parathyroid hormone receptor-like GPCRs family B (8–11), suggesting that the GLP-2 receptor might also be found within this subfamily. Initial attempts at GLP-2 receptor cloning by conventional screening of cDNA libraries at low stringency with a combination of GLP-1 and glucagon receptor cDNA probes were not successful. Accordingly, we next used a combined approach of reverse transcription–PCR and hybridization screening followed by expression

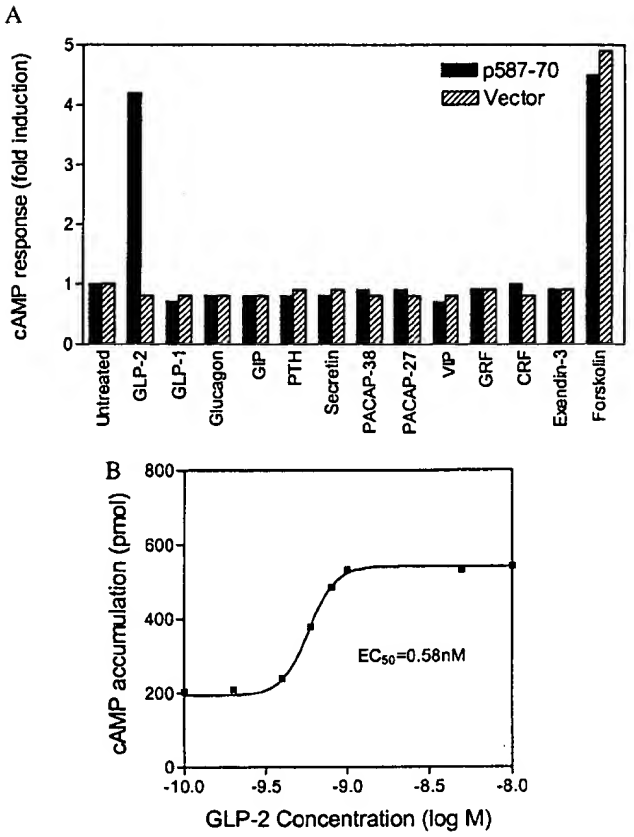

FIG. 2. Ligand-selective and concentration-dependent cAMP response to rat GLP-2 in transiently lipofected COS cells. (*A*) Ligand specificity of cAMP response to GLP-2. cAMP response to peptide analogs of family 2 GPCR ligands was determined in COS cells transiently lipofected with the GLP-2R expression vector p587-70 or the parental pcDNA3 expression vector. GLP-2 concentration was 1 nM; all other peptides were used at 10 nM. A similar profile of peptide specificity was observed with the human GLP-2R (data not shown). (*B*) Concentration–response curve for cAMP accumulation in response to synthetic rat GLP-2 in rG2R cells stably expressing GLP-2R.

analysis of candidate cDNAs. As described in *Methods and Materials*, this strategy resulted in the isolation of a 2,537-bp rat GLP-2R cDNA insert encoding a 550-aa putative family B GPCR (Fig. 1). Hydropathy analysis of the GLP-2R amino acid sequence revealed a typical 7-transmembrane topology plus a hydrophobic amino-terminal signal peptide (data not shown). The GLP-2R gene product belongs to the GLP-1/glucagon/GIP receptor gene subfamily. Conserved features include a possible signal-peptide cleavage site between Val-64 and Thr-65, potential *N*-glycosylation sites within the amino-terminal putative extracellular domain, and six cysteine residues conserved in the mature GLP-1, GIP, and glucagon receptor amino-terminal domains (Fig. 1). Two putative alternative translation initiation codons, Met-1 and Met-42, were found amino-terminal to the first transmembrane domain in the rat GLP-2R. Functional analysis of Met-1 and Met-42 site-directed mutants showed they were functionally identical (unpublished results), consistent with signal-peptide removal predicted to yield an identical 486-aa mature polypeptide. Overlapping regions of the hypothalamus and duodenum/jejunum cDNA clones encoded homologous polypeptide fragments. Moreover, no evidence was obtained for differential splicing of intestinal GLP-2R RNA from an RNase protection assay employing two nonoverlapping probes derived from GLP-2R cDNA, which together spanned 387 of the 550 GLP-2R codons (data not shown). Additionally, sequencing of

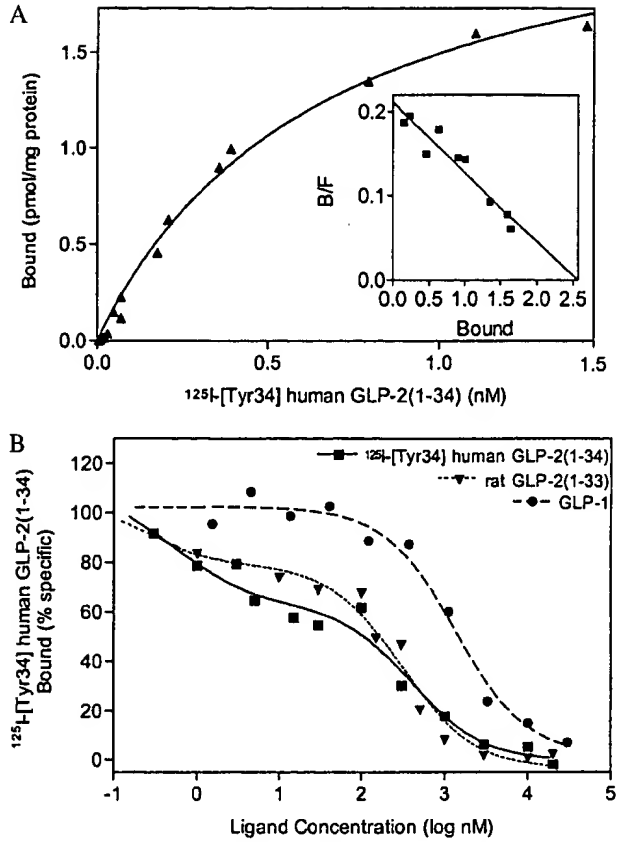

FIG. 3. Binding of ^{125}I -[Tyr-34]GLP-2 to cell membranes prepared from rG2R cells stably transfected with GLP-2R cDNA. (*A*) Saturation isotherms of the specific binding of ^{125}I -[Tyr-34]GLP-2 to membranes. Results shown are representative of six independent experiments, each conducted in triplicate. From Scatchard analysis (*Inset*), maximal-binding B_{max} was estimated at 1,839 fmol/mg protein, and a K_d value of 0.57 nM was obtained. (*B*) Competition binding of ^{125}I-[Tyr-34]GLP-2 binding to cell membranes in the presence of unlabeled peptides. Data are shown for the concentration-dependent inhibition of ^{125}I-[Tyr-34]GLP-2 binding (200 pmol) to GLP-2R by various peptide analogs from at least two independent experiments conducted in triplicate. Inhibitory constants (K_i) were estimated by using GRAPHPAD PRISM.

the full-length human GLP-2R cDNAs confirmed the identity of hypothalamic and gastrointestinal GLP-2Rs (Fig. 1).

To assess whether the predicted GLP-2R sequence encodes a functional GLP-2 receptor, a GLP-2R expression construct, p587-70, was transiently transfected into COS cells. Because related family B GPCRs show functional coupling to cAMP production mediated by the heterotrimeric G protein Gs, cAMP accumulation was measured after incubation with GLP-2. Treatment of GLP-2R-transfected COS cells with 1 nM GLP-2 resulted in a 4-fold rise in cAMP levels relative to untreated cells, approximately equal to the response seen with 10 μM forskolin (Fig. 2*A*). Treatment with 10 nM GLP-1, glucagon, GIP, exendin-3, and seven other family B GPCR ligands did not induce cAMP production in p587-70-transfected cells. Control cells transfected with vector DNA alone failed to respond to any of the peptides tested, including GLP-2, though forskolin did induce cAMP production.

With a stable GLP-2R episomal expression cell line, rG2R, greater than 20-fold cAMP induction routinely was achieved with 1 or 10 nM GLP-2 (data not shown), confirming the potent induction of cAMP accumulation by GLP-2. In contrast, the nontransfected 293-EBNA cells showed no response to GLP-2. The EC_{50} of the cAMP response to GLP-2 in rG2R cells was 0.58 nM (Fig. 2*B*). The presence of saturable, specific, ligand-selective GLP-2-binding sites on these cells was shown by using a radioiodinated, C-terminally extended GLP-2 analog, ^{125}I-[Tyr-34]GLP-2 (Fig. 3*A*). From Scatchard analysis of the saturation isotherms, a B_{max} value of 1,839 fmol/mg of protein and a K_d of 0.57 nM were obtained for the radioligand. Mock-transfected cells showed no specific binding to the radioligand (data not shown). Competition-binding studies with rat GLP-2 revealed a high-affinity site (K_i = 0.06 nM) and a low-affinity site (K_i = 259 nM) (Fig. 3*B*). In contrast, no high-affinity GLP-1 sites were observed in the transfected GLP-2R/rG2R clone. K_i values determined for GLP-1, glucagon, and GIP peptides were 928, 500, and 765 nM, respectively. Although the binding and Scatchard data may reflect, in part, a degree of receptor overexpression, the functional studies and binding data provide firm evidence for a cDNA that encodes a functional high-affinity, ligand-selective GLP-2 receptor.

The human GLP-2R polypeptide showed 81.6% similarity to rat GLP-2R (Fig. 1). Functional expression of the cloned human GLP-2R conferred a functional response to GLP-2 and the appearance of high-affinity ligand-binding sites on 293-EBNA cells, similar to data obtained with the rat GLP-2R (data not shown). Furthermore, the cloned human GLP-2 receptor exhibited the same profile of peptide-binding specificity (Fig. 2*A* and unpublished data) as the rat receptor. The gene encoding human GLP-2R was identified by screening an arrayed BAC library of human genomic DNA (Genome Systems, St. Louis), confirmed by sequencing, and mapped to chromosome 17p13.3 by fluorescence *in situ* hybridization analysis (data not shown).

A quantitative ribonuclease protection assay method was used to determine the tissue distribution of rat GLP-2R RNA because no signals were detected on multitissue Northern blots. GLP-2R RNA levels were highest in jejunum, followed by duodenum, ileum, colon, and stomach, whereas expression was undetectable in seven other tissues (Table 1). This expression pattern is clearly concordant with previously reported functional responses to GLP-2 in duodenum (12, 13), jejunum, ileum (5, 12, 14, 15), and colon (12, 16, 17); in contrast, no proliferative or histological changes were seen after GLP-2 treatment in spleen, heart, kidney, lung, or brain (18). Thus, GLP-2R expression is detected in known GLP-2 target tissues. This observation, together with the functional data from

Table 1. Quantitative GLP-2R RNA distribution in various rat tissues determined by RNase protection

Tissue	F1 quantitation, copies per μg total RNA	β-Actin quantitation, copies per μg total RNA	GLP-2R/-actin, ratio
Jejunum	11,900	15,500,000	76.8×10^{-5}
Duodenum	9,150	85,700,000	10.7×10^{-5}
Ileum	7,490	51,400,000	14.6×10^{-5}
Colon	4,150	19,800,000	21.0×10^{-5}
Stomach	1,530	23,600,000	6.48×10^{-5}
Brain	<600	40,600,000	$<1.48 \times 10^{-5}$
Heart	<600	6,600,000	$<9.09 \times 10^{-5}$
Kidney	<600	14,900,000	$<4.03 \times 10^{-5}$
Liver	<600	16,700,000	$<3.59 \times 10^{-5}$
Lung	<600	38,500,000	$<1.56 \times 10^{-5}$
Muscle	<600	4,600,000	$<13.0 \times 10^{-5}$
Spleen	<600	44,800,000	$<1.34 \times 10^{-5}$

Total RNA (50 μg) from rat tissues or sense-strand cRNA standards was hybridized to radiolabeled antisense cRNA probes prepared *in vitro* from GLP-2R cDNA (F1) or actin cDNA. After RNase digestion as described in *Methods and Materials*, protected probe was precipitated, electrophoresed, and quantitated by PhosphorImage analysis relative to the standard curve obtained from sense-strand cRNA. RNA quantitation is expressed as copy number per μg of total RNA. GLP-2R RNA copy number was detectable to a lower limit of 30,000 copies per 50 μg sample, setting the limit of detection shown above for nongastrointestinal tissues.

Table 2. *In vitro* and *in vivo* activity profiles of selected peptide analogs of GLP-2

Peptide	K_i, nM[a*]		EC_{50}, nM†	E_{max}, %†‡	*In vivo* activity§
	High-affinity	Low-afinity			
rGLP-2(1-33)¶	0.06 ± 0.00	259 ± 46	1.00 ± 0.2	100 ± 0	Active
N-Ac-rGLP-2(1-33)	—	140 ± 2	20.8 ± 0.1	80.1 ± 7	Partially active
[Arg-1]rGLP-2(−1-33)	NA	*n**	901 ± 41	69.0 ± 2	Inactive
[Arg-34]rGLP-2(1-34)	ND	*n*§	3.1 ± 0.3	105 ± 10	Active
[Tyr-34]hGLP-2	0.56 ± 0.3	255 ± 7	1.4 ± 0.1	113 ± 5	Active
rGLP-2(2-33)	—	876 ± 147	210 ± 22	109 ± 8	Inactive
rGLP-2(3-33)	—	251 ± 8	10.7 ± 0.8	115 ± 5	Inactive
rGLP-2(1-29)	0.30 ± 0.00	584 ± 19	3.60 ± 0.4	102 ± 8	Partially active
[Thr-7 insertion]	—	977 ± 470	1,100 ± 30	76 ± 4.0	Inactive
[Gly-2]GLP-2(1-33)	—	126 ± 5	2.0 ± 0.2	103 ± 6	Active
hGLP-2(1-33)¶	1.7 ± 0.4	596 ± 9	1.3 ± 0.20	99 ± 10.6	Active
Glucagon	—	500 ± 332	NA	NA	Inactive
GLP-1(7-36)amide	—	928 ± 1	NA	NA	Inactive
GIP	—	765 (n = 1)	NA	NA	Inactive

NA, not active—no detectable binding; ND, not determined.
*n = 2, except where indicated.
†n = 3.
‡Relative to 100 nM rGLP-2(1-33), n = 3.
§Relative to vehicle-treated control animals, n = 4 or greater. *In vivo* activity is based on changes in small bowel wet weight after 14-day treatment as described in *Methods and Materials*.
¶rGLP-2(1-33) is native rat GLP-2 peptide; hGLP-2(1-33) is native human GLP-2 peptide.

experiments with cloned GLP-2R cDNA, suggests that this receptor mediates the intestinotrophic actions of GLP-2.

Pharmacological support for this hypothesis was obtained from parallel *in vivo/in vitro* studies of GLP-2 analogs containing simple changes in sequence and length (Table 2). Carboxyl-terminal extension analogs bound and activated GLP-2R and retained *in vivo* activity whereas those with amino-terminal extensions lost both activities. Analogs with blocked amino- or carboxyl-terminal residues displayed diminished *in vivo* activity and GLP-2R activation. Insertion of a Thr residue between GLP-2 residues 6 and 7 resulted in loss of activity *in vivo* and *in vitro*. Truncation of the carboxyl-terminus to a 29-residue peptide (analogous to glucagon) reduced but did not eliminate *in vivo* or *in vitro* activities. Interestingly, truncation of one or two amino-terminal residues abolished *in vivo* activity but did not completely eliminate binding or the GLP-2R cAMP response. Taken together, a clear correspondence was revealed between the structural requirements for GLP-2R binding and activation and the *in vivo* intestinotrophic activity of GLP-2, providing additional evidence that the GLP-2R isolated here and the intestinotrophic GLP-2 receptor mediating GLP-2 action *in vivo* are synonymous.

Enteroglucagon synthesis long has been associated with a humoral adaptive response to massive small bowel resection, in which hyperplasia and elongation of jejunal villi are seen (19–22). Proglucagon-derived GLP-2 is detectable in plasma from fasted rats and humans and rises 1.5- to 3.6-fold after feeding (23). Moreover, the intestinotrophic efficacy of GLP-2 has been shown after administration by i.p., i.m., or s.c. routes (14), as well as by coinfusion in parenterally fed rats (12). Thus, it is likely that circulating GLP-2 mediates adaptive changes in the villus-absorptive area in the small intestine. The cloning and characterization of a GLP-2 receptor expressed in the gastrointestinal tract qualifies GLP-2, like GLP-1, glucagon, and GIP, as a bona fide endocrine hormone and should facilitate the discovery of novel pharmacologic agents with similar functional activity. The expression of GLP-2R in hypothalamus also raises the possibility of as yet undescribed role(s) for this intestinotrophic hormone in the central nervous system.

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1. Drucker, D. J. (1998) *Diabetes* **47,** 159–169.
2. McGregor, G. P., Goke, R. & Goke, B. (1998) *Exp. Clin. Endocrinol. Diabetes* **106,** 25–28.
3. Tsai, C.-H., Hill, M., Asa, S. L., Brubaker, P. L. & Drucker, D. J. (1997) *Am. J. Physiol.* **273,** E77–E84.
4. Nomenclature Committee of the International Union of Biochemistry (NC-IUB) (1986) *J. Biol. Chem.* **261,** 13–17.
5. Drucker, D. J., Ehrlich, P., Asa, S. L. & Brubaker, P. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7911–7916.
6. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1998) *Current Protocols in Molecular Biology* (Wiley, New York).
7. Fehmann, H.-C., Goke, R. & Goke, B. (1995) *Endocrine Rev.* **16,** 390–410.
8. Thorens, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8641–8645.
9. MacNeil, D. J., Occi, J. L., Hey, P. J., Strader, C. D. & Graziano, M. P. (1994) *Biochem. Biophys. Res. Commun.* **198,** 328–334.
10. Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kuijper, J. L., Sheppard, P. O., Sprecher, C. A., *et al.* (1993) *Science* **259,** 1614–1616.
11. Yasuda, K., Inagaki, N., Yamada, Y., Kubota, A., Seino, S. & Seino, Y. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1556–1562.
12. Chance, W. T., Foley-Nelson, T., Thomas, I. & Balasubramaniam, A. (1997) *Am. J. Physiol.* **273,** G559–G563.
13. Brubaker, P. L., Izzo, A., Hill, M. & Drucker, D. J. (1997) *Am. J. Physiol.* **272,** E1050–E1058.
14. Tsai, C.-H., Hill, M. & Drucker, D. J. (1997) *Am. J. Physiol.* **272,** G662–G668.
15. Cheeseman, C. I. (1997) *Am. J. Physiol.* **273,** R1965–R1971.
16. Drucker, D. J., Deforest, L. & Brubaker, P. L. (1997) *Am. J. Physiol.* **273,** G1252–G1262.
17. Litvak, D. A., Hellmich, M. R., Evers, B. M., Banker, N. A. & Townsend, C. M., Jr. (1998) *J. Gastrointest. Surg.* **2,** 146–150.
18. Tsai, C.-H., Hill, M., Asa, S. L., Brubaker, P. L. & Drucker, D. J. (1997) *Am. J. Physiol.* **273,** E77–E84.
19. Buchan, A. M. J., Griffiths, C. J., Morris, J. F. & Polak, J. M. (1985) *Gastroenterology* **88,** 8–12.
20. Rountree, D. B., Ulshen, M. H., Selub, S., Fuller, C. R., Bloom, S. R., Ghatei, M. A. & Lund, P. K. (1992) *Gastroenterology* **103,** 462–468.
21. Gornacz, G. E., Ghatei, M. A. & Al-Mukthar, M. Y. T. (1984) *Dig. Dis. Sci.* **29,** 1041–1049.
22. Taylor, R. G. & Fuller, P. J. (1994) *Baillieres Clin. Endocrinol. Metab.* **8,** 165–183.
23. Brubaker, P. L., Crivici, A., Izzo, A., Ehrlich, P., Tsai, C.-H. & Drucker, D. J. (1997) *Endocrinology* **138,** 4837–4843.